



Article scientifique

Article

2004

Published version

Open Access

This is the published version of the publication, made available in accordance with the publisher's policy.

A role for Cajal bodies in the final steps of U2 snRNP biogenesis

Nesic, Dobrila; Tanackovic, Goranka; Kraemer, Angela

How to cite

NESIC, Dobrila, TANACKOVIC, Goranka, KRAEMER, Angela. A role for Cajal bodies in the final steps of U2 snRNP biogenesis. In: Journal of cell science, 2004, vol. 117, n° Pt 19, p. 4423–4433. doi: 10.1242/jcs.01308

This publication URL: <https://archive-ouverte.unige.ch/unige:17542>

Publication DOI: [10.1242/jcs.01308](https://doi.org/10.1242/jcs.01308)

A role for Cajal bodies in the final steps of U2 snRNP biogenesis

Dobrila Nesic*, Goranka Tanackovic and Angela Krämer[‡]

Department of Cell Biology, Faculty of Sciences, University of Geneva, 30, quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland

*Present address: Osteoarticular Research Group, Institute of Pathology, University of Bern, Murtenstrasse 31, CH-3010 Bern, Switzerland

[‡]Author for correspondence (e-mail: angela.kraemer@cellbio.unige.ch)

Accepted 10 May 2004

Journal of Cell Science 117, 4423-4433 Published by The Company of Biologists 2004

doi:10.1242/jcs.01308

Summary

The biogenesis of Sm-type small nuclear ribonucleoproteins (snRNPs) involves the export of newly transcribed small nuclear RNAs (snRNAs) to the cytoplasm, assembly with seven common proteins and modification at the 5' and 3' termini. Binding of snRNP-specific proteins and snRNA modification complete the maturation process. This is thought to occur after reimport of the core snRNPs into the nucleus. The heterotrimeric splicing factor SF3a converts a pre-mature 15S U2 snRNP into the functional 17S particle. To analyze cellular aspects of this process, we studied domains in SF3a60 and SF3a66 that are required for their localization to nuclear speckles. Regions in SF3a60 and SF3a66 that mediate the binding to SF3a120 are necessary for nuclear import of the proteins, suggesting that the SF3a heterotrimer forms in the

cytoplasm. SF3a60 and SF3a66 deleted for zinc finger domains required for the incorporation of SF3a into the U2 snRNP are nuclear, indicating that the 17S U2 snRNP is assembled in the nucleus. However, these proteins show an aberrant nuclear distribution. Endogenous SF3a subunits colocalize with U2 snRNP in nuclear speckles, but cannot be detected in Cajal bodies, unlike core U2 snRNP components. By contrast, SF3a60 and SF3a66 lacking the zinc finger domains accumulate in Cajal bodies and are diffusely distributed in the cytoplasm, suggesting a function for Cajal bodies in the final maturation of the U2 snRNP.

Key words: Cajal body, Localization, SF3a, snRNP biogenesis, Splicing factor

Introduction

The U1, U2, U4, U5 and U6 small nuclear ribonucleoproteins (snRNPs) play key roles in the splicing of the vast majority of pre-mRNAs by assembling the primary transcript into a catalytically active complex, the spliceosome (Will and Lührmann, 1997; Nilsen, 1998). Each snRNP consists of a uridine-rich small nuclear RNA (snRNA) and seven common (Sm) proteins (B/B', D1, D2, D3, E, F and G) or, in the case of the U6 snRNP, seven Sm-like (Lsm) proteins. The majority of the U4 and U6 snRNAs are found in one particle, the U4/U6 snRNP, which associates with the U5 snRNP to function in splicing. In addition to the Sm and Lsm proteins, each snRNP contains particle-specific proteins.

Maturation of the mammalian Sm-type snRNPs involves the export of snRNAs to the cytoplasm and re-import of core snRNPs into the nucleus (Will and Lührmann, 2001). U1, U2, U4 and U5 snRNAs are transcribed by RNA polymerase II and acquire a monomethylated m⁷G cap. Upon export of newly synthesized snRNAs to the cytoplasm, the Sm proteins interact with the conserved Sm binding site. This process is facilitated by a protein complex containing the survival of motor neurons (SMN) protein implicated in spinal muscular atrophy (Paushkin et al., 2002). The association of Sm proteins with a given snRNA is a prerequisite for 3' end trimming and hypermethylation of the cap generating the characteristic 5' m₃^{2,2,7}G (Will and Lührmann, 2001). The Sm domain and the m₃G cap form a bipartite nuclear localization signal (NLS) that is required for reimport of the newly assembled core snRNPs into the nucleus.

Within the nucleus, the bulk of the snRNPs accumulate in interchromatin granule clusters, which appear by fluorescence microscopy as nuclear speckles (Lamond and Spector, 2003). The speckled pattern is superimposed onto a diffuse nucleoplasmic staining, probably representing snRNPs actively involved in the splicing of nascent transcripts. A smaller fraction of snRNAs, Sm proteins and some particle-specific proteins are also found in Cajal bodies (CBs). In contrast, splicing factors that are not associated with any snRNP are excluded from these structures (Matera, 1999; Gall, 2000; Ogg and Lamond, 2002). CBs are present in varying number and size in many but not all animal and plant cells and are dynamic structures that assemble and disassemble during the cell cycle and move within the nucleus. Apart from snRNP components and p80 coilin, an unambiguous marker for CBs, these structures contain nucleolar factors including small nucleolar (sno) RNPs, components of the basal transcription machinery and cell cycle factors. In addition, CBs associate with specific gene loci, such as histone and U2 snRNP gene clusters. Thus, roles for CBs in transport, regulation of gene expression and assembly of macromolecular complexes have been proposed.

A function for CBs in the biogenesis of snRNPs is supported by several recent studies. First, upon import into the nucleus, newly formed U snRNPs pass through CBs and only later accumulate in speckles, but following mitosis, snRNPs localize to speckles immediately (Sleeman and Lamond, 1999; Sleeman et al., 2001; Ogg and Lamond, 2002). Second, proteins that promote the assembly of the U4/U6 and

U4/U6.U5 snRNPs are enriched in CBs (Makarova et al., 2002; Stanek et al., 2003). Third, the snRNAs of fully matured snRNPs contain numerous base and sugar modifications, which are guided by CB-specific small RNAs containing sequences complementary to conserved sites of 2'-O-methylation and pseudouridylation in the snRNAs (Kiss, 2001; Carmo-Fonseca, 2002). These modifications are introduced into snRNAs in CBs (Jády et al., 2003).

Binding of snRNP-specific proteins is required for the production of mature snRNPs that are active in splicing (Krämer, 1996; Will and Lührmann, 2001). Unlike other stages in snRNP biogenesis, the cellular site(s) of this event and the timing with respect to other maturation steps are unknown. Several U1-specific proteins and the U2-specific A' and B'' proteins are transported into the nucleus independently of their cognate snRNAs (Feeney and Zieve, 1990; Jantsch and Gall, 1992; Kambach and Mattaj, 1992; Kambach and Mattaj, 1994; Romac et al., 1994; Hetzer and Mattaj, 2000), implying that the final assembly occurs after import of the core snRNPs into the nucleus. Moreover, modification of the U2 snRNA is required for the binding of several U2-specific proteins in *Xenopus* oocytes (Yu et al., 1998; Zhao and Yu, 2004). Given recent evidence that snRNAs are modified in CBs (Jády et al., 2003), it is conceivable that particle-specific proteins associate with the core snRNPs either during or after their passage through the CBs.

In the present study we have analyzed the localization of U2 snRNP-specific proteins to gain further information regarding the final steps in snRNP biogenesis. The U2 snRNP is well suited for such analysis because the localization of the core components is known; many, if not all, U2-specific proteins have been identified; and the assembly of the mature U2 snRNP has been established biochemically. The mature 17S U2 snRNP, which is active in splicing, consists of the U2 snRNA, the Sm proteins, the U2-specific proteins A' and B'' and splicing factors SF3a and SF3b (Behrens et al., 1993a; Behrens et al., 1993b; Brosi et al., 1993a; Krämer et al., 1999). U2A' and U2B'' are stably associated with the core U2 snRNP in a 12S particle, whereas SF3a and SF3b are bound to the U2 snRNP in a salt-labile fashion. In vitro, the mature U2 snRNP can be reconstituted from purified components in a two-step pathway. Binding of SF3b to the 12S U2 generates an intermediate particle of 15S, and further association of SF3a results in the formation of the functional 17S U2 snRNP (Brosi et al., 1993a; Krämer et al., 1999). SF3a consists of three subunits (SF3a60, SF3a66 and SF3a120) (Brosi et al., 1993b), whereas SF3b comprises eight subunits (Das et al., 1999; Krämer et al., 1999; Will et al., 2001; Will et al., 2002). Immunoaffinity-purified 17S U2 snRNP contains several additional proteins that are not present in SF3a or SF3b; one SF3b protein, SF3b125, is absent from the 17S U2 snRNP (Will et al., 2002).

Components of the 12S U2 snRNP are distributed in speckles and in the nucleoplasm and are also enriched in CBs (Lamond and Carmo-Fonseca, 1993; Spector, 1993). In contrast, although integral components of the 17S U2 snRNP, SF3a subunits and SF3b155 have been detected in the nucleoplasm and in speckles, but neither SF3a120 nor SF3b155 were found to accumulate in CBs (Chiara et al., 1994; Hong et al., 1997; Schmidt-Zachmann et al., 1998; Eilbracht and Schmidt-Zachmann, 2001; Will et al., 2002). This suggests that CB-associated U2 snRNPs are not fully matured. On the

other hand, SF3b125 and hPrp5p (a 17S U2 protein absent from purified SF3b) are not only detected in speckles, but also in CBs, and functions for these proteins in the final maturation of the U2 snRNP have been proposed (Will et al., 2002).

A previous structure-function analysis demonstrated that the N-terminal portions of SF3a60 and SF3a66 are required for interaction with SF3a120 and formation of the SF3a heterotrimer (Nesic and Krämer, 2001). Additionally, the zinc finger domains of SF3a60 and SF3a66 were found to be indispensable for the incorporation of SF3a into the U2 snRNP. With this information at hand, we analysed the contribution of these domains to the intracellular localization of SF3a60 and SF3a66. We show here that sequences in SF3a60 and SF3a66 required for the binding to SF3a120 target the proteins to the nucleus and that nuclear import of SF3a60 and SF3a66 is independent of an association with the U2 snRNP. Moreover, deletion of the zinc finger domains of SF3a60 and SF3a66 leads to an accumulation of the proteins in CBs, accompanied by their depletion from nuclear speckles, supporting a role for CBs in the final steps of U2 snRNP biogenesis. Finally, our data suggest that a highly conserved region in SF3a60 contributes to a speckled localization.

Materials and Methods

Cloning procedures

The coding sequence of GFP carrying a S65T mutation was inserted into the *EcoRI* site of pSG5 (Stratagene) containing a modified multiple cloning site, thus generating vector pSG5-GFP. The coding sequences of the SF3a subunits and truncated derivatives (except for GFP-3a60-C3 and C4) were cloned into the Klenow-treated *KpnI* site of pSG5-GFP. Full-length sequences of the SF3a subunits were derived from the original cDNA clones in pBluescript with appropriate restriction enzymes (Krämer et al., 1994; Krämer et al., 1995; Nesic and Krämer, 2001). Inserts of SF3a60 proteins with N-terminal and internal deletions were generated by PCR as described (Nesic and Krämer, 2001). The inserts of GFP-3a60-ΔZn and ΔSAP correspond to 3a60-Δ4 and Δ1, respectively, of Nesic and Krämer (Nesic and Krämer, 2001). 3a60-C3 and C4 were derived from 3a60-FL in pSG5-GFP by restriction enzyme digestion and insertion into the Klenow-treated *KpnI* site of pEGFP-C1 (Clontech).

The insert encoding 3a66-N (termed 3a66-C2) (Nesic and Krämer, 2001) was cleaved from pSG5-GFP-3a66-FL by digestion with *BamHI* and *StuI*, followed by Klenow treatment and cloning into the Klenow-treated *KpnI* site of pSG5-GFP. The inserts encoding 3a66-C (previously termed 3a66-N1) (Nesic and Krämer, 2001) and 3a66ΔZn were derived from pFastBac HT constructs with appropriate restriction enzymes and cloned into pSG5-GFP as above. Correct cloning was verified by restriction analysis and DNA sequencing. In addition, proteins translated in vitro from pSG5-GFP plasmids migrated in SDS polyacrylamide gels with the expected sizes.

The plasmid for expression of GFP-tagged estrogen receptor was a gift from Didier Picard.

Cell culture and transient transfections

HeLa cells were grown as monolayers in minimum essential medium supplemented with 2 mM L-glutamine, 10% FCS and 50 IU/ml penicillin and 50 mg/ml streptomycin (GIBCO). For localisation studies cells were transfected at a confluency of ~50% by calcium phosphate precipitation (Sambrook et al., 1989) and analyzed 48 hours after transfection. Cells used for the experiment shown in Fig. 7D and for immunoprecipitation were transfected by electroporation as described in the Easyject Plus (Equibio) manual.

Immunoprecipitation

Small-scale nuclear extracts (Krämer and Keller, 1990) were prepared from HeLa cells transfected with GFP-3a60-FL, GFP-3a66-FL, GFP-3a120-FL, GFP-3a60ΔZn and GFP-3a66-ΔZn 48 hours post-transfection. Aliquots (40 µg of total protein in 100 µl 20 mM HEPES-KOH, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA and 0.5 mM dithiothreitol) were pre-cleared by binding to 30 µl Protein G-Sepharose (Amersham) for 2 hours at 4°C, followed by overnight incubation at 4°C with 25 µl Protein G-Sepharose-coupled anti-GFP (Molecular Probes). The beads were washed seven times in 1 ml 50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 0.05% Nonidet-P40 and 0.5 mM dithiothreitol and bound material was eluted with 2× SDS sample buffer. Proteins were separated in a 10% SDS polyacrylamide gel, transferred to nitrocellulose (Kyhse-Anderson, 1984), and detected with anti-3a66, anti-3a120, anti-3a60 (see below) and guinea pig anti-3b155 (Schmidt-Zachmann et al., 1998), appropriate secondary antibodies and the Supersignal kit (Pierce). The same membrane was used for all antibodies, which were stripped from the membrane by incubation for 5 minutes in 0.2 M NaOH and 15 minutes in PonceauS (Sigma).

Indirect immunofluorescence and in situ hybridization

Cells grown on 10-mm square glass coverslips were washed twice in PBS and permeabilized with 0.5% Triton X-100 in CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES, 3 mM MgCl₂, 1 mM EGTA, pH 6.8) (Fey et al., 1986) for 1 minute on ice, fixed in 3.7% formaldehyde in CSK buffer for 15 minutes at room temperature and rinsed with PBS. Cells expressing GFP-tagged proteins were fixed in methanol at -20°C for 4 minutes. This protocol allowed for better resolution of nuclear structures probably owing to an extraction of loosely bound GFP-tagged proteins.

For immunofluorescence, cells were incubated for 20 minutes with primary antibodies diluted in PBS, washed three times for 5 minutes each in PBS and incubated for 20 minutes with appropriate secondary antibodies conjugated to FITC or rhodamine. The cells were washed three times for 5 minutes each in PBS and mounted in Mowiol. Cells shown in Fig. 7D were incubated for 30 minutes each with primary and secondary antibodies and all washing and incubation steps were performed in PBS containing 0.2% Nonidet-P40. The following primary antibodies were used: rabbit anti-SF3a60 (Krämer et al., 1994), monoclonal anti-SF3a66 (Brosi et al., 1993b), rabbit anti-SF3a120 (Krämer et al., 1995), monoclonal anti-SC35 (Fu and Maniatis, 1990), monoclonal anti-Sm (Y12) (Lerner et al., 1981), monoclonal anti-U2B'' (4G3) (Habets et al., 1989), rabbit anti-p80-coilin (Bohmann et al., 1995) and mouse anti-p80-coilin (Almeida et al., 1998).

In situ hybridization was performed essentially as described (Carmo-Fonseca et al., 1991). Cells grown on coverslips were washed in 6× SSPE (Sambrook et al., 1989) and incubated for 15 minutes with 0.5 µg/µl yeast tRNA in 6× SSPE and 5× Denhardt's solution, followed by incubation for 5 minutes in the same buffer supplemented with 0.1 pmol of a biotinylated oligoribonucleotide complementary to the 5' end of U2 snRNA (Carmo-Fonseca et al., 1991). The cells were washed three times for 15 minutes in 6× SSPE at room temperature and rinsed twice for 5 minutes in avidin wash buffer (20 mM HEPES-KOH, pH 7.9, 150 mM KCl, 0.05% Tween 20). Subsequently, cells were incubated for 5 minutes with streptavidin-conjugated FITC (Vector) in freshly prepared 20 mM HEPES-KOH, pH 7.9, 250 mM KCl, 0.5 mM DTT and 1% BSA. The cells were washed three times for 5 minutes in avidin wash buffer, once in PBS-0.1% Tween, incubated with antibodies as described above and mounted in Mowiol.

Fluorescence microscopy

Fluorescence microscopy was performed with a Zeiss inverted fluorescence microscope (Axiovert TV 135) using a 100× Planapo

objective. Images were taken at 1317×1035 pixels with a cooled charge-coupled device camera (model CH250, Photometrics). FITC and rhodamine channels were recorded individually with the software package IPLab spectrum V2.3 (Signal Analytics) and processed with Adobe Photoshop 5.0 (Adobe system). Pseudo-coloured images of the FITC and rhodamine signals were generated and superimposed. The images shown in Fig. 7D were obtained with a 63× Planapo objective and taken at 1360×1036 pixels with a Retiga EX camera (Q Imaging). FITC, rhodamine and UV channels were recorded with Openlab software (Improvision) and processed with Adobe Photoshop 7.0.

Results

Endogenous SF3a subunits colocalize in nuclear speckles and the nucleoplasm, but are excluded from CBs

Components of the 12S U2 snRNP, i.e. the U2 snRNA, the Sm proteins and the U2 snRNP-specific proteins A' and B'', localize to nuclear speckles and CBs (Lamond and Carmo-Fonseca, 1993; Spector, 1993). It has previously been reported that SF3a60 and SF3a66 are present in speckles, however their presence in CBs was not directly assessed (Chiara et al., 1994; Hong et al., 1997). Will et al. (Will et al., 2002) did not detect SF3a120 in CBs, although it is an integral component of the mature and splicing-competent 17S U2 snRNP (Brosi et al., 1993a; Nesic and Krämer, 2001). To test whether SF3a60 and SF3a66 were similarly underrepresented in CBs we performed immunofluorescence studies of HeLa cells. In agreement with the previously reported distribution, the antibodies used in our study detected all SF3a subunits in the nucleus with exclusion of the nucleoli (Fig. 1A,B). Prominent staining of irregularly shaped structures resembling nuclear speckles was accompanied by diffuse nucleoplasmic staining. The enhanced nucleoplasmic staining with anti-SF3a60 and anti-SF3a120 compared to anti-SF3a66 may be caused by the polyclonal nature of these antibodies. All subunits colocalized in the same nuclear structures as evident from computer-generated overlays of the individual images (Fig. 1A,B, right panels). Counterstaining of cells with anti-SF3a60 and an antibody against SC35, a splicing protein with an almost exclusively speckled localization (Fu and Maniatis, 1990; Spector et al., 1991), confirmed that the bright structures decorated by anti-SF3a antibodies represent nuclear speckles (Fig. 1C). Double immunofluorescence with anti-SF3a66 and anti-p80-coilin showed the typical speckled pattern for SF3a66, whereas p80-coilin was restricted to CBs, which appeared green in the overlay (Fig. 1D). Thus, similar to SF3a120, SF3a66 appears to be absent from CBs. Comparison of the distribution of SF3a60 and U2B'' (or Sm proteins) revealed colocalization in speckles, but SF3a60 was not detected in CBs, which were decorated by anti-U2B'' and appeared red in the computer-generated overlay (Fig. 1E; data not shown). Identical results were obtained with combinations of anti-SF3a120 and anti-Sm antibodies (data not shown). Finally, the localization of SF3a66 was compared with that of U2 snRNA, which was visualized by in situ hybridization of HeLa cells with a complementary 2'-OMe oligoribonucleotide. U2 snRNA was detected in speckles and CBs, whereas SF3a66 was excluded from CBs, evident from the red staining of the CBs in the overlay of the two images (Fig. 1F). Similarly, SF3a60 and SF3a120 colocalized with U2 snRNA in speckles but not in CBs (data not shown). Together these results demonstrate that the SF3a

subunits, like other non-snRNP splicing factors, localize to nuclear speckles and exhibit a diffuse distribution in the nucleoplasm but cannot be detected in CBs. The possibility that the epitopes of the SF3a subunits are not accessible in CBs can

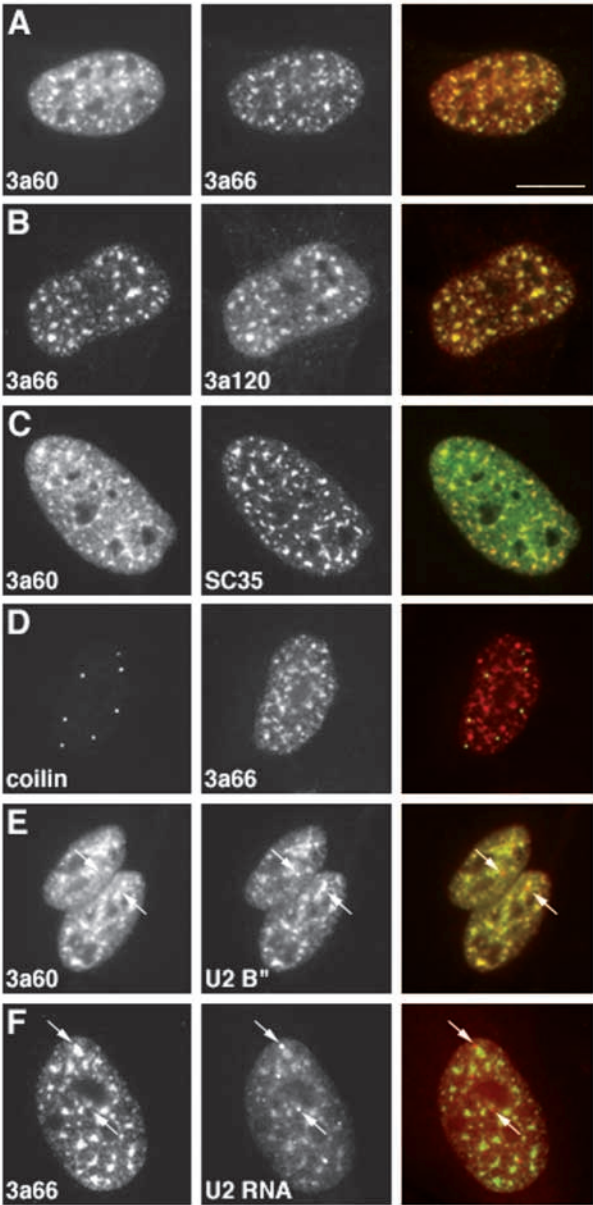


Fig. 1. Comparison of the localization of endogenous SF3a subunits with other splicing components. (A-E) HeLa cells were permeabilized with Triton X-100, fixed with paraformaldehyde and treated with antibodies against the following proteins: (A) SF3a60 and SF3a66; (B) SF3a66 and SF3a120; (C) SF3a60 and SC35; (D) p80-coilin and SF3a66; (E) SF3a60 and U2 B''. (F) Cells were hybridized with a 2' O-methyl oligoribonucleotide complementary to U2 snRNA and immunostained with an anti-SF3a66 antibody. Images shown in all left panels were obtained after staining with FITC-coupled secondary antibodies. Images shown in middle panels were stained with rhodamine-coupled secondary antibodies. The right panels show computer-generated overlays of the two images. CBs appear in green in panel (D), and red in panels (E) and (F). Arrows in (E) and (F) indicate the absence (left panels) or presence (middle and right panels) of CBs. Bar, 10 μ m.

be excluded, because anti-SF3a antibodies readily detect overexpressed SF3a subunits that accumulate in these structures (data not shown; see below). Together, these results suggest that the majority of the U2 snRNPs present in CBs are not associated with SF3a and thus are not fully matured.

Transiently expressed SF3a subunits are present in nuclear speckles, but also accumulate in CBs

To study sequences that contribute to the proper nuclear localization of SF3a60 and SF3a66, we transiently expressed individual SF3a subunits as fusions to the green-fluorescent protein (GFP) in HeLa cells and monitored their localization by fluorescence of GFP. We had previously shown that glutathione S-transferase- or His₆-tagged SF3a proteins interacted with one another, and His₆-tagged subunits bound to the 15S U2 snRNP in vitro (Nesic and Krämer, 2001). To ensure that the corresponding interactions in vivo were not impaired by the GFP tag, individual GFP-tagged SF3a subunits were transiently expressed in HeLa cells. Small-scale nuclear extracts were prepared 48 hours post-transfection. Immunoprecipitation with an anti-GFP antibody followed by western blotting with anti-SF3a antibodies revealed that GFP-tagged full-length SF3a subunits or GFP-tagged SF3a60 and SF3a66 with zinc finger domain deletions associated with the

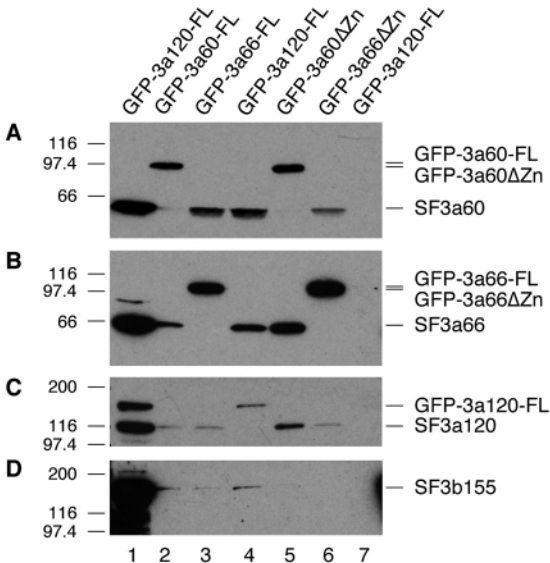


Fig. 2. Analysis of interactions of GFP-tagged SF3a subunits with endogenous subunits and the 15S U2 snRNP. (A-D) HeLa cells were transiently transfected with plasmids encoding full-length GFP-tagged SF3a subunits (FL) or GFP-SF3a60 and GFP-SF3a66 deleted for their zinc finger domains (Δ Zn) as indicated above the figure. SF3a complexes were precipitated from small-scale nuclear extracts prepared 48 hours post-transfection with Protein G-Sepharose-coupled anti-GFP (lanes 2-6). Bound proteins were analysed by western blotting for the presence of SF3a60 (A), SF3a66 (B), SF3a120 (C) and SF3b155 (D). Lane 1 shows the extract prepared from cells overexpressing GFP-3a120-FL as an example for the input of the immunoprecipitation reactions. Lane 7 shows the same extract treated with Protein G-Sepharose in the absence of anti-GFP. Positions of precipitated proteins are indicated on the right. The migration of molecular mass standards (in kDa) is shown on the left.

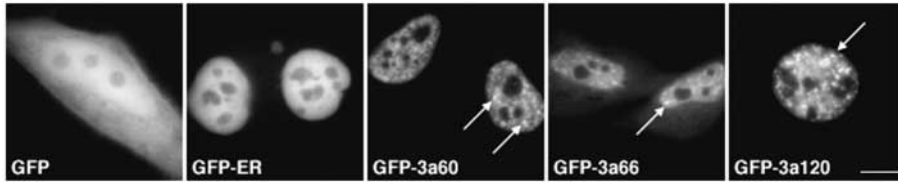


Fig. 3. Localization of transiently expressed SF3a subunits fused to GFP in live cells. HeLa cells were transiently transfected with plasmids encoding GFP, GFP-ER and SF3a subunits fused to GFP as indicated. Fluorescence was monitored in living cells. The arrows point to bright foci. Bar, 10 μ m.

remaining two endogenous subunits (Fig. 2, lanes 2-6). Importantly, the anti-GFP antibody did not precipitate the endogenous counterparts of the transiently expressed proteins, demonstrating that the co-precipitated, endogenous SF3a subunits are exclusively bound to the GFP-tagged proteins. SF3b155, a component of SF3b (that binds to the U2 snRNP prior to SF3a, but does not interact with SF3a in the absence of the U2 snRNP) (Krämer et al., 1999), co-precipitated with full-length GFP-SF3a subunits, indicating that these proteins also interact with the 15S U2 snRNP (lanes 2-4). In contrast, SF3b155 is not precipitated after expression of GFP-tagged SF3a60 and SF3a66 lacking the zinc finger domains that are essential for incorporation of SF3a into the mature U2 snRNP (lanes 5 and 6). The rather low amount of co-precipitated SF3b155 is probably caused by a slightly increased salt concentration and the presence of detergent during the washing procedure, which may contribute to a partial dissociation of SF3a from the U2 snRNP (Krämer et al., 1999). Together, these results demonstrate that the GFP tag does not interfere with interactions between the SF3a subunits or with the binding of SF3a to the 15S U2 snRNP *in vivo*. Moreover, the GFP tag probably does not affect the movement of the SF3a subunits in the nucleus, because GFP-SF3a subunits responded to transcription inhibition similar to other splicing factors (Lamond and Spector, 2003) (data not shown).

We next tested whether the full-length, GFP-tagged SF3a subunits localized to the same structures as the endogenous proteins. GFP or GFP fused to the estrogen receptor (GFP-ER) were expressed as controls (Fig. 3). GFP was distributed throughout the cell, whereas GFP-ER was confined to the nucleus consistent with previous reports (Picard et al., 1990). The GFP-tagged SF3a subunits were present in bright, irregularly shaped structures resembling nuclear speckles and showed a diffuse nucleoplasmic distribution, similar to the results obtained by immunofluorescence (Fig. 1). In some images bright foci were observed (indicated by arrows in Fig. 3) and high levels of expression sometimes caused weak cytoplasmic fluorescence.

A localization of transiently expressed GFP-SF3a subunits in speckles was confirmed by counterstaining of transfected HeLa cells with anti-SC35 after fixation in cold methanol to remove proteins that are not tightly associated with nuclear structures (Fig. 4A,C; data not shown). To examine whether the bright foci occasionally observed after transient expression of GFP-SF3a subunits represented CBs, HeLa cells transfected with GFP-3a60 and GFP-3a66 were stained with anti-p80-coilin, anti-Sm and anti-U2B^{''}. The foci decorated by these antibodies corresponded to GFP fluorescence in the same structures, confirming the presence of GFP-SF3a proteins in CBs (Fig. 4B,D; data not shown). In addition, bright foci containing GFP-3a60 or GFP-3a66 were not stained by anti-SC35 (Fig. 4A,C). As SC35 has not been found in CBs (Fu and Maniatis, 1990; Spector et al., 1991), this result further

supports the accumulation of overexpressed SF3a subunits in these structures. To exclude the possibility that the presence of the GFP tag caused an accumulation of the GFP-tagged SF3a subunits in CBs, full-length SF3a60 without a tag was transiently expressed in HeLa cells. The distribution of endogenous and transiently expressed proteins was monitored with anti-SF3a60 and compared with the localization of SC35, p80-coilin, Sm proteins and U2B^{''}. In contrast to the results presented in Fig. 1, where anti-SF3a60 detected the endogenous protein in speckles and the nucleoplasm, CBs were clearly decorated upon overexpression of SF3a60 (data not

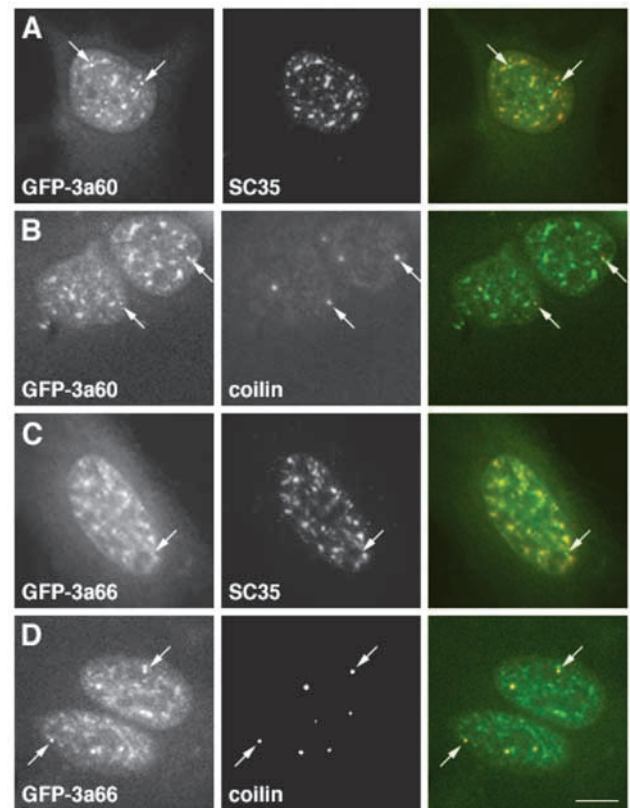


Fig. 4. Presence of transiently expressed GFP-3a60 and GFP-3a66 in nuclear speckles and CBs. (A-D) The localization of transiently expressed GFP-3a60 and GFP-3a66 was analyzed after fixation of HeLa cells in cold methanol. The left panels show the fluorescence of GFP-3a60 (A and B) and GFP-3a66 (C and D). Images in the middle panels were obtained after immunostaining with anti-SC35 (A and C) or anti-p80-coilin (B and D) and rhodamine-coupled secondary antibodies. The right panels represent computer-generated overlays of the images shown on the left and in the middle. Colocalization of GFP-3a60 and GFP-3a66 with p80-coilin in CBs (indicated by arrows) appears in yellow in (B) and (D). CBs stained by GFP-3a60 and GFP-3a66 but not by SC35 appear green in the overlays in (A) and (C). Bar, 10 μ m.

shown). Together, these results show that although transiently expressed SF3a subunits localize to nuclear speckles similar to their endogenous counterparts, overexpression of the proteins can lead to an accumulation in CBs.

The N-terminal portion of SF3a60 is necessary for localization in nuclear speckles and deletion of the zinc finger domain results in an accumulation in CBs

Our previous results indicated that the N terminus of SF3a60 mediates the interaction with SF3a120, a C-terminal zinc finger domain is required for incorporation into the 17S U2 snRNP and both regions are necessary for the function of SF3a60 in pre-spliceosome assembly (Nesic and Krämer, 2001). To test

the contribution of these domains to the intracellular localization of SF3a60, cDNAs encoding GFP-tagged truncated or internally deleted versions of SF3a60 (Fig. 5A) were transiently transfected into HeLa cells. Protein localization was analyzed by fluorescence in living cells (Fig. 5B) and, for a more detailed analysis of an association with

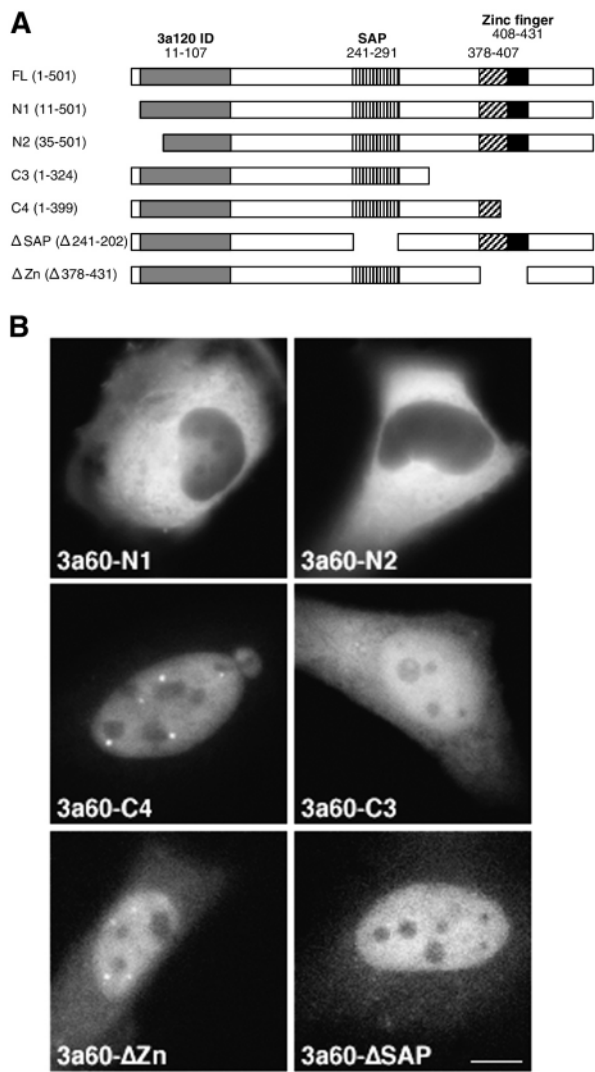


Fig. 5. Analysis of domains in SF3a60 required for nuclear and subnuclear localization in live cells. (A) Schematic representation of recombinant SF3a60 proteins fused to GFP. Amino acids present in, or deleted from the recombinant proteins are given in parentheses after the names of the proteins. Conserved regions are indicated above the diagram and numbered according to the amino acids in full-length SF3a60. (B) GFP-3a60 proteins with N- or C-terminal or internal deletions (as indicated) were transiently expressed in HeLa cells. Fluorescence was monitored in live cells. Bar, 10 μm.

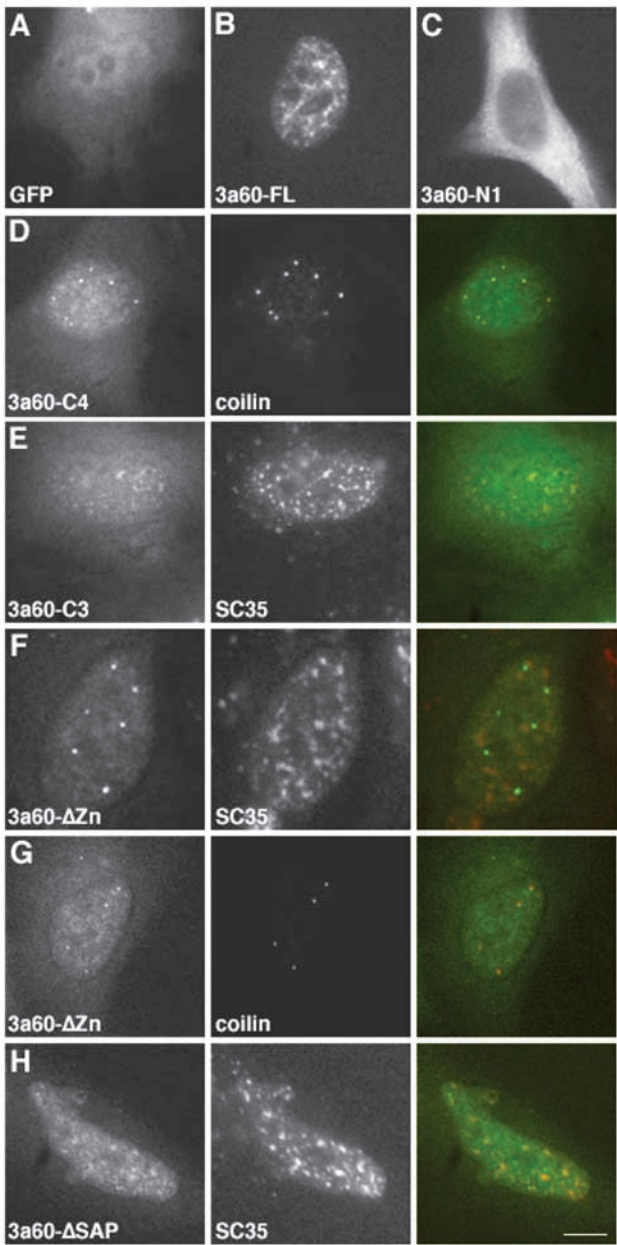


Fig. 6. Comparison of the distribution of transiently expressed GFP-3a60 proteins with SC35 and p80-coilin in fixed cells. (A-H) HeLa cells were transiently transfected with plasmids encoding GFP (A), GFP-3a60-FL (B), GFP-3a60-N1 (C), GFP-3a60-C4 (D), GFP-3a60-C3 (E), GFP-3a60-ΔZn (F and G) and GFP-3a60-ΔSAP (H). Cells were fixed in cold methanol and immunostained with anti-p80-coilin (D and G) or anti-SC35 (E, F and H). The images in (A-C) and the left panels of (D-H) show fluorescence of GFP. The middle panels of (D-H) show immunolocalization of p80-coilin or SC35, and the right panels represent computer-generated overlays of the two images. Bar, 10 μm.

particular nuclear structures, after fixation of transfected cells in methanol (Fig. 6). Deletion of the N-terminal 10 or 34 amino acids of SF3a60 (GFP-3a60-N1 and N2, respectively) resulted in a complete loss of nuclear localization (Fig. 5B, Fig. 6C), indicating an essential function of the SF3a60 N terminus in nuclear targeting.

GFP-3a60-C4 lacking 102 C-terminal amino acids was evenly distributed throughout the nucleoplasm of living HeLa cells but also accumulated in a few bright foci (Fig. 5B). In methanol-fixed cells a speckled nuclear distribution, comparable to that of transiently expressed full-length SF3a60 was barely detectable (cf. Fig. 6B,D). The relatively diffuse nuclear signal of GFP-3a60-C4 was, however, clearly different from the residual nuclear staining of transiently expressed GFP (Fig. 6A). Counterstaining with anti-p80-coilin identified the bright foci as CBs (Fig. 6D). In addition, a faint cytoplasmic signal of GFP-3a60-C4 was observed in images of fixed cells. Further deletion of C-terminal sequences (GFP-3a60-C3) resulted in diffuse nuclear and increased cytoplasmic fluorescence in living cells and, compared to GFP-3a60-C4, the enrichment in bright foci was lost (Fig. 5B). In methanol-fixed cells GFP-3a60-C3 was never detected in bright foci and the nuclear signal varied from diffuse to a low degree of residual staining in speckles (Fig. 6E; data not shown). SF3a60-C4 and C3 lack the zinc finger domain that is required for U2 snRNP binding (Nesic and Krämer, 2001). We therefore questioned whether an internal deletion of this domain would suffice for the aberrant localization of SF3a60. GFP-3a60- Δ Zn displayed a diffuse nucleoplasmic distribution in living cells and accumulated in several bright foci (Fig. 5B). In fixed cells, the fluorescence in nuclear speckles was reduced to background levels when compared with the immunostaining by anti-SC35 (Fig. 6F), whereas the protein was clearly present in CBs, as shown by counterstaining with anti-p80-coilin (Fig. 6G). Thus, deletion of the zinc finger domain appears to be responsible for the accumulation of SF3a60 in CBs and a concomitant depletion of the protein from nuclear speckles.

We also tested the localization of GFP-3a60 Δ SAP, which lacks an internal sequence that is highly conserved in metazoan SF3a60 and shows similarity to the SAF-A/B, Acinus and PIAS (SAP) motif (Aravind and Koonin, 2000). Our previous analysis did not reveal any function for this sequence (Nesic and Krämer, 2001), but given its high level of conservation it was of interest to test whether the SAP motif might play a role in the localization of SF3a60. Indeed, although GFP-3a60 Δ SAP was mainly nucleoplasmic in living cells (Fig. 5B), analysis of fixed cells revealed a reduced fluorescence in nuclear speckles and a more pronounced diffuse nucleoplasmic staining compared to GFP-3a60-FL (cf. Fig. 6H,B).

In summary, these results indicate that the N terminus of SF3a60 is essential for nuclear targeting. Sequences located at the C terminus may contribute to nuclear localization, because we consistently observed some cytoplasmic staining of SF3a60 with C-terminal deletions. However, the cytoplasmic staining could also be caused by a high overexpression of the proteins. In addition, C-terminal sequences are essential for SF3a60 localization in speckles. Most importantly, deletion of the zinc finger domain resulted in an accumulation of the protein in CBs and a depletion from nuclear speckles. Thus, SF3a60, which is

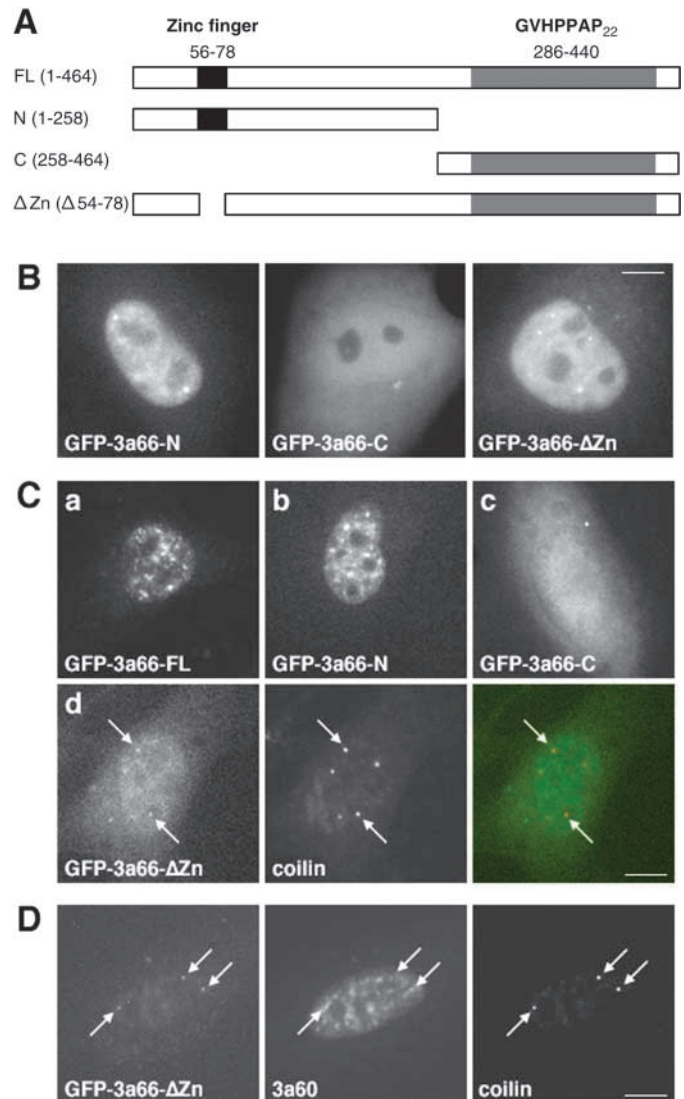


Fig. 7. Analysis of domains in SF3a66 required for nuclear and subnuclear localization. (A) Schematic representation of recombinant SF3a66 proteins fused to GFP. Amino acids present in, or deleted from the recombinant proteins are given in parentheses after the names of the proteins. Conserved regions are indicated above the diagram and numbered according to the amino acids in full-length SF3a66. (B) GFP-tagged 3a66-N, -C and - Δ Zn were transiently expressed in HeLa cells and visualized by fluorescence in living cells. (C) HeLa cells were transiently transfected with plasmids encoding GFP-SF3a66 proteins as indicated. Fluorescence was monitored after fixation of cells with cold methanol. Cells in (d) were immunostained with anti-p80-coilin (middle panel); the right panel represents the computer-generated overlay. (D) HeLa cells were transiently transfected with GFP-3a66- Δ Zn. Proteins (as indicated) were visualised after fixation of cells with cold methanol. SF3a60 was visualised by immunofluorescence with anti-3a60 and AMCA-coupled anti-rabbit antibodies, p80-coilin with a mouse anti-p80-coilin and Texas Red-coupled anti-mouse antibodies. Arrows point to CBs. Bar, 10 μ m.

impaired in binding to the U2 snRNP, localizes aberrantly in the nucleus. Finally, the SAP motif appears to contribute to a speckled localization of SF3a60.

The N-terminal half of SF3a66 is necessary and sufficient for nuclear targeting, and deletion of the zinc finger domain causes an accumulation in CBs

Our previous results indicated that the N-terminal half of SF3a66 is sufficient for interaction with SF3a120, assembly of the 17S U2 snRNP and pre-spliceosome formation, whereas the C-terminal half, consisting of 22 heptad repeats, is dispensable for these functions (Nesic and Krämer, 2001). In addition, a zinc finger domain close to the N-terminus of the protein is required for the incorporation of SF3a66 into the U2 snRNP but not for binding to SF3a120.

To test which sequences in SF3a66 are necessary for proper intracellular localization, HeLa cells were transfected with plasmids encoding the N- and C-terminal halves of SF3a66 (GFP-3a66-N and -C, respectively) or with SF3a66 lacking the zinc finger domain (GFP-3a66-ΔZn; Fig. 7A). In images of live cells GFP-3a66-N was exclusively nuclear (Fig. 7B). Immunostaining of methanol-fixed cells confirmed that the protein colocalized with SC35, indicating that the N-terminal half of SF3a66 is sufficient for targeting to nuclear speckles (Fig. 7C; data not shown). Similar to the transiently expressed full-length protein, GFP-3a66-N also accumulated in several bright foci in some experiments (cf. Fig. 3, Fig. 7B). GFP-3a66-C was evenly distributed throughout the cell, which was evident both in live and fixed cells (Fig. 7B,C). No preference for any cellular structure was apparent in fixed cells and the overall fluorescence was somewhat decreased (panel c), similar to the fluorescence of GFP alone (cf. Fig. 6A). The uniform distribution of GFP-3a66-C throughout the cell could in principle be due to a M_r of 52,000 for the fusion protein, which is below the limit of ~60 kDa for diffusion through nuclear pores (Görlich and Kutay, 1999). On the other hand, the M_r of GFP-3a66-N is 60,600 but the protein is exclusively nuclear, indicating that the N-terminal half of SF3a66 harbours a sequence(s) required for nuclear targeting. Deletion of the SF3a66 zinc finger domain (GFP-3a66-ΔZn) led to a diffuse staining in the nucleus, a weak cytoplasmic signal and the appearance of bright foci in living cells (Fig. 7B). In fixed cells, GFP-3a66-ΔZn appeared to be absent from nuclear speckles, and immunostaining with anti-p80-coilin confirmed that the foci corresponded to CBs (Fig. 7C, panel d). In addition, GFP-3a66-ΔZn co-localized with anti-Sm and anti-U2B' in CBs but not in speckles (data not shown).

Deletion of the zinc finger domains of SF3a60 and SF3a66 does not abolish their ability to bind SF3a120 (Nesic and Krämer, 2001) (Fig. 2). It might therefore be expected that endogenous subunits accumulate in CBs together with GFP-3a60-ΔZn or GFP-3a66-ΔZn. When HeLa cells were transfected with GFP-3a66-ΔZn, not only was this protein detected in CBs, but endogenous SF3a60 (Fig. 7D) and SF3a120 (data not shown) were also present. The localisation of SF3a60 to nuclear speckles remained largely unaffected when compared to untransfected cells (cf. Fig. 1).

In conclusion, the N-terminal portion of SF3a66, comprising all sequences required for SF3a66 function in pre-spliceosome assembly (Nesic and Krämer, 2001), is necessary and sufficient for correct nuclear distribution. Deletion of the zinc finger domain, which is essential for the integration of SF3a66 into the 17S U2 snRNP, led to an almost complete depletion of the protein from nuclear speckles and an accumulation in CBs, similar to that observed with GFP-3a60-ΔZn. In addition,

endogenous SF3a subunits concentrate in CBs in cells expressing GFP-3a66-ΔZn. Together with the observation that GFP-3a66-ΔZn binds to endogenous SF3a60 and SF3a120 (Fig. 2), this result demonstrates that it is the SF3a heterotrimer and not only the overexpressed protein that is retained in CBs, when binding to the U2 snRNP is compromised. Thus, the zinc finger domains in both SF3a60 and SF3a66 play a major role in targeting SF3a to its proper intranuclear location.

Discussion

The three SF3a subunits are essential for the conversion in vitro of an intermediate 15S U2 snRNP into the 17S particle active in splicing (Behrens et al., 1993a; Brosi et al., 1993a; Krämer et al., 1999). To complement our previous structure-function analysis of SF3a60 and SF3a66 (Nesic and Krämer, 2001), we have now identified domains that mediate the proper localization of these proteins in HeLa cells.

Import of SF3a into the nucleus

SF3a60 proteins with deletions of 10 or 34 N-terminal amino acids were excluded from nuclei, indicating that sequences at the N terminus of SF3a60 are essential for nuclear targeting. Although the N-terminal 26 amino acids (of the sequence METILEQRRYHEEKERLMDVMKEM) are highly conserved during evolution, this region does not contain sequences resembling known NLSs, such as the classical basic SV40-type or bipartite NLSs, the glycine-rich M9 domain of hnRNP A1, the KNS domain of hnRNP K, basic NLSs in several ribosomal proteins or histone H1, NLSs in the viral M protein, and unusual NLSs in the U1A and U2B' proteins (for a review, see Mattaj and Englmeier, 1998; Hetzer and Mattaj, 2000; Glodowski et al., 2002). Thus, nuclear targeting of SF3a60 may rely on a novel type of NLS. On the other hand, the N terminus of SF3a60 mediates the interaction with SF3a120. The major site for SF3a120 binding in vitro is located between amino acids 35 and 107 of SF3a60 and the N-terminal 34 amino acids are required for a stable interaction (Nesic and Krämer, 2001). Given the correlation between sequences that mediate SF3a120 binding and nuclear localization of SF3a60, an attractive possibility is that SF3a60 is targeted to the nucleus in association with SF3a120.

SF3a60 proteins deleted for C-terminal sequences showed varying degrees of cytoplasmic localization (Fig. 5B and Fig. 6). Because overexpression of the full-length proteins also resulted in a low level of cytoplasmic staining in some experiments, it is difficult to assess whether the C-terminal sequences contribute to nuclear targeting. However, cytoplasmic fluorescence was most pronounced and reproducible for GFP-3a60-C3, the most extensive C-terminal deletion mutant. Therefore, if C-terminal sequences of SF3a60 are involved in nuclear targeting, these sequences are probably located between residues 324 and 399. We would like to note that all SF3a60 proteins analyzed exhibiting cytoplasmic fluorescence have sizes above the limit for diffusion through nuclear pores (Görlich and Kutay, 1999), suggesting that these proteins do not passively move between the nucleus and the cytoplasm.

The N-terminal half of SF3a66, excluding the zinc finger domain, is sufficient for nuclear import of the protein. Despite

an extremely high phylogenetic conservation of the N-terminal ~220 amino acids, neither this region, nor the remainder of the protein contain an identifiable NLS. Thus, similar to SF3a60, nuclear import of SF3a66 may be mediated by an unusual NLS or SF3a66 also enters the nucleus in association with SF3a120. Interestingly, we found that SF3a120 deleted for sequences required for interaction with SF3a60 and SF3a66 was confined to the nucleus (data not shown), suggesting that SF3a120 contains a true NLS. Hence, we favour the possibility that the SF3a subunits associate with one another in the cytoplasm and traverse the nuclear pore together.

A comparable example of a splicing factor that is imported into the nucleus as a heterodimer is U2AF, consisting of two subunits (U2AF⁶⁵ and U2AF³⁵), each of which contains an arginine/serine-rich (RS) domain (Gama-Carvalho et al., 2001). RS domains have been shown to target proteins to the nucleus and to nuclear speckles (Li and Bingham, 1991; Hedley et al., 1995; Cáceres et al., 1997). Interestingly, deletion of the RS domain in either of the U2AF subunits does not compromise nuclear localization; however when both RS domains are missing, the proteins remain cytoplasmic. In addition, upon deletion of the U2AF³⁵ interaction domain, nuclear import of U2AF⁶⁵ becomes dependent on the presence of its RS domain. Based on these results it was proposed that the U2AF heterodimer is imported into the nucleus as a complex, with the RS domains serving as redundant NLSs (Gama-Carvalho et al., 2001). Other examples of spliceosomal proteins that employ a 'piggy-back' mechanism for nuclear import are the U2 snRNP-specific protein U2B'' (Kambach and Mattaj, 1994) and the cap binding protein CBP20 (Izaurre et al., 1995).

Although the signal that targets the SF3a heterotrimer to the nucleus remains to be identified, our results clearly indicate that the association of SF3a60 and SF3a66 with the U2 snRNP is a nuclear event. SF3a60 and SF3a66 proteins deleted for their zinc finger domains, which mediate binding to the U2 snRNP (Nesic and Krämer, 2001), are nuclear. Therefore, the SF3a subunits are imported into the nucleus independently of the U2 snRNP, as shown for U2A' and U2B'' (Kambach and Mattaj, 1994). Nuclear targeting of U1 snRNP-specific proteins has also been reported to occur independently of the interaction with the U1 snRNA (Jantsch and Gall, 1992; Kambach and Mattaj, 1992; Romac et al., 1994). Moreover, an association of SF3a with SF3b prior to nuclear import is unlikely, because SF3a does not interact with SF3b in the absence of the U2 snRNP (Krämer et al., 1999).

Cajal bodies as sites for the final steps in U2 snRNP maturation

All components of the 12S U2 snRNP (U2 snRNA, Sm proteins, U2A' and U2B'') are present in nuclear speckles and CBs (Lamond and Carmo-Fonseca, 1993; Spector, 1993), whereas the SF3a subunits and SF3b155 have only been detected in speckles (Schmidt-Zachmann et al., 1998; Will et al., 2002) (see also our data). The bulk of the SF3a subunits and SF3b155 are associated with the U2 snRNP in nuclear extracts (Brosi et al., 1993a; Will et al., 2002). This implies that the U2 snRNP population in nuclear speckles represents mature 17S particles, whereas the majority of the U2 snRNPs in CBs are not fully assembled. Upon transient expression of

full-length SF3a subunits in HeLa cells, we sometimes observed an accumulation of the proteins in CBs in addition to the typical speckled distribution. The enrichment of the SF3a subunits in CBs was not caused by the presence of the GFP tag, because transiently expressed, untagged SF3a60 was also detected in CBs (data not shown). Similarly, GFP-3a60-C4 and -ΔZn as well as GFP-3a66-ΔZn were enriched in CBs, but otherwise displayed a diffuse nucleoplasmic distribution without concentration in speckles. In contrast, GFP-3a60-C3 did not accumulate in CBs, despite an abnormal nuclear distribution, making it unlikely that overexpression of SF3a60 or SF3a66 sequences per se results in an enrichment of the proteins in CBs. The most likely interpretation of our data is that GFP-3a60-C4, -ΔZn and GFP-3a66-ΔZn accumulate in CBs due to a failure in associating with the U2 snRNP (Nesic and Krämer, 2001), thus blocking further distribution of the proteins to speckles and sites of splicing, which is consistent with the observation that these proteins do not concentrate in nuclear speckles. The failure to detect endogenous SF3a subunits in CBs in untransfected cells could be explained by rapid movement of newly matured 17S U2 snRNPs from CBs to sites of splicing or storage, which is supported by comparably short residence times of snRNP components in CBs (Dundr et al., 2004). Transiently expressed full-length SF3a subunits would accumulate in CBs if high overexpression of the proteins caused an imbalance in the concentration of endogenous and transiently expressed SF3a subunits compared to the concentration of the 12S U2 snRNP and/or SF3b, which are both required for the incorporation of SF3a into the 17S U2 snRNP (Brosi et al., 1993a; Krämer et al., 1999). Such a scenario is underscored by estimates suggesting approximately equimolar amounts of the U2 snRNP and SF3a in the nucleus (Brosi et al., 1993b).

Together, our results strongly implicate CBs in the final steps of U2 snRNP biogenesis. This notion is supported by several previous findings. First, after import from the cytoplasm newly assembled core snRNPs pass through CBs before they are detected in speckles (Sleeman and Lamond, 1999). Second, among novel constituents of SF3b and the 17S U2 snRNP, hPrp5p was identified as a protein associated with the 17S U2 snRNP, but absent from SF3b, whereas SF3b125 appears to dissociate from SF3b once the 17S U2 snRNP is formed (Will et al., 2002). Both proteins are members of the DEAD box family of RNA unwindases/RNAPases and based on their differential association with SF3b and the 17S U2 snRNP, Will et al. (Will et al., 2002) proposed functions for hPrp5p and SF3b125 in U2 snRNP maturation. In contrast to the SF3a subunits and SF3b155, hPrp5p and SF3b125 are not only detected in nuclear speckles but also in CBs. Third, U2 snRNA modification is a prerequisite for the formation of the 17S U2 snRNP in *Xenopus* oocytes (Yu et al., 1998; Zhao and Yu, 2004) and this event takes place in CBs (Jády et al., 2003).

The result that GFP-3a60-C3 did not accumulate in CBs was somewhat unexpected, because, similar to GFP-3a60-C4, it lacks the zinc finger domain and does not associate with the U2 snRNP (Nesic and Krämer, 2001). Fluorescence varied from a diffuse appearance in the nucleus to very low residual staining in speckles. A diffuse nuclear localization of GFP-3a60-C3 could correlate with a loss in the ability of the protein to be targeted to CBs. Indeed, upon nuclear import transiently expressed Sm proteins are diffusely distributed in the nucleus

prior to localization in CBs (Sleeman and Lamond, 1999) and the same could be true for SF3a. Whether the sequences encompassing residues 324-399 of SF3a60 could be involved in directing SF3a to CBs requires further study.

Our results suggest that the SAP motif may be involved in the localization of SF3a60 to nuclear speckles. Deletion of this region caused a partial depletion of the mutant protein from speckles and an increase in diffuse nucleoplasmic staining when compared to GFP-3a60-FL. Interestingly, neither U2 snRNP binding nor SF3a60 function in spliceosome assembly in vitro were affected in SF3a60 lacking the SAP motif (Nesic and Krämer, 2001). Thus, this region could be required to target the mature U2 snRNP to nuclear speckles. The SAP motif has been identified in more than 200 proteins with functions in the regulation of transcription and chromatin structure (Aravind and Koonin, 2000). Among these is the scaffold attachment factor SAF-B, which interacts with RNA polymerase II and several members of the SR family of splicing proteins, and colocalizes with SC35 in speckles (Nayler et al., 1998). In addition, SF3b145/SAP145, a component of SF3b and the mature U2 snRNP (Gozani et al., 1996), contains an N-terminal SAP motif. It has been suggested that the SAP motif may target proteins to specific chromosomal locations, thereby contributing to the coupling of transcription with splicing and other processes (Aravind and Koonin, 2000). Domains that have been implicated in targeting of proteins to nuclear speckles are RS domains and RNA-recognition motifs in SR proteins (Li and Bingham, 1991; Hedley et al., 1995; Cáceres et al., 1997) and a region enriched in threonine/proline dipeptides in SF3b155 (Eilbracht and Schmidt-Zachmann, 2001). Neither of the SF3a subunits contain any of these sequences, thus it is possible that the SAP motif functions as a signal for targeting SF3a to speckles.

Based on our results we propose a model in which the SF3a subunits assemble into a stable complex in the cytoplasm and are imported into the nucleus in the form of the heterotrimer. The signal that targets SF3a to the nucleus remains to be determined and could either be present in SF3a120 or formed upon assembly of the SF3a heterotrimer. Once in the nucleus, SF3a is targeted to CBs and incorporated into the U2 snRNP to form the mature 17S particle. Endogenous SF3a does not accumulate in CBs; thus, it appears that the final step in U2 snRNP biogenesis is rapid, and mature particles move from CBs to sites of active splicing or storage as soon as they are formed. Finally, targeting of the mature U2 snRNP to speckles may involve the SAP motif in SF3a60 and possibly additional sequences present in other components of the U2 snRNP.

We thank Angus Lamond for anti-p80-coilin antibodies and oligonucleotides complementary to U2 snRNA, Joan Steitz for the Y12 cell line, Walther von Venrooij for anti-U2 B'', Xiang-Dong Fu for anti-SC35, and Didier Picard for an expression plasmid of GFP-tagged estrogen receptor. We are grateful to Anne-Marie Tourmel for technical assistance and Holly Goodson, Karl Matter and Jeffrey Patton for helpful discussions. This work was supported by a grant from the Swiss National Science Foundation to A.K.

References

Almeida, F., Saffrich, R., Ansorge, W. and Carmo Fonseca, M. (1998). Microinjection of anti-coilin antibodies affects the structure of coiled bodies. *J. Cell Biol.* **142**, 899-912.

Aravind, L. and Koonin, E. V. (2000). SAP – a putative DNA-binding motif involved in chromosomal organization. *Trends Biochem. Sci.* **25**, 112-114.

Behrens, S. E., Galisson, F., Legrain, P. and Lührmann, R. (1993a). Evidence that the 60-kDa protein of 17S U2 small nuclear ribonucleoprotein is immunologically and functionally related to the yeast PRP9 splicing factor and is required for the efficient formation of prespliceosomes. *Proc. Natl. Acad. Sci. USA* **90**, 8229-8233.

Behrens, S. E., Tyc, K., Kastner, B., Reichelt, J. and Lührmann, R. (1993b). Small nuclear ribonucleoprotein (RNP) U2 contains numerous additional proteins and has a bipartite RNP structure under splicing conditions. *Mol. Cell. Biol.* **13**, 307-319.

Bohmann, K., Ferreira, J., Santama, N., Weis, K. and Lamond, A. I. (1995). Molecular analysis of the coiled body. *J. Cell Sci.* **19**, 107-113.

Brosi, R., Gröning, K., Behrens, S.-E., Lührmann, R. and Krämer, A. (1993a). Interaction of mammalian splicing factor SF3a with U2 snRNP and relation of its 60-kD subunit to yeast PRP9. *Science* **262**, 102-105.

Brosi, R., Hauri, H. P. and Krämer, A. (1993b). Separation of splicing factor SF3 into two components and purification of SF3a activity. *J. Biol. Chem.* **268**, 17640-17646.

Cáceres, J. F., Misteli, T., Sreter, G. R., Spector, D. L. and Krainer, A. R. (1997). Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. *J. Cell Biol.* **138**, 225-238.

Carmo-Fonseca, M. (2002). New clues to the function of the Cajal body. *EMBO Rep.* **3**, 726-727.

Carmo-Fonseca, M., Tollervey, D., Pepperkok, R., Barabino, S. M., Merdes, A., Brunner, C., Zamore, P. D., Green, M. R., Hurt, E. and Lamond, A. I. (1991). Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery. *EMBO J.* **10**, 195-206.

Chiara, M. D., Champion-Arnaud, P., Buoli, M., Nadal-Ginard, B. and Reed, R. (1994). Specific protein-protein interactions between the essential mammalian spliceosome-associated proteins SAP 61 and SAP 114. *Proc. Natl. Acad. Sci. USA* **91**, 6403-6407.

Das, B. K., Xia, L., Palandjian, L., Gozani, O., Chyung, Y. and Reed, R. (1999). Characterization of a protein complex containing spliceosomal proteins SAPs 49, 130, 145, and 155. *Mol. Cell. Biol.* **19**, 6796-6802.

Dundr, M., Hebert, M. D., Karpova, T. S., Stanek, D., Xu, H., Shpargel, K. B., Meier, U. T., Neugebauer, K. M., Matera, A. G. and Misteli, T. (2004). In vivo kinetics of Cajal body components. *J. Cell Biol.* **164**, 831-842.

Eilbracht, J. and Schmidt-Zachmann, M. S. (2001). Identification of a sequence element directing a protein to nuclear speckles. *Proc. Natl. Acad. Sci. USA* **98**, 3849-3854.

Feeney, R. J. and Zieve, G. W. (1990). Nuclear exchange of the U1 and U2 snRNP-specific proteins. *J. Cell Biol.* **110**, 871-881.

Fey, E. G., Krochmalnic, G. and Penman, S. (1986). The nonchromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J. Cell Biol.* **102**, 1654-1665.

Fu, X.-D. and Maniatis, T. (1990). Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature* **343**, 437-441.

Gall, J. G. (2000). Cajal bodies: the first 100 years. *Annu. Rev. Cell Dev. Biol.* **16**, 273-300.

Gama-Carvalho, M., Carvalho, M. P., Kehlenbach, A., Valcárcel, J. and Carmo-Fonseca, M. (2001). Nucleocytoplasmic shuttling of heterodimeric splicing factor U2AF. *J. Biol. Chem.* **276**, 13104-13112.

Glodowski, D. R., Petersen, J. M. and Dahlberg, J. E. (2002). Complex nuclear localization signals in the matrix protein of vesicular stomatitis virus. *J. Biol. Chem.* **277**, 46864-46870.

Görlich, D. and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Dev. Biol.* **15**, 607-660.

Gozani, O., Feld, R. and Reed, R. (1996). Evidence that sequence-independent binding of highly conserved U2 snRNP proteins upstream of the branch site is required for assembly of spliceosomal complex A. *Genes Dev.* **10**, 233-243.

Habets, W. J., Hoet, M. H., de Jong, B. A., van der Kemp, A. and van Venrooij, W. J. (1989). Mapping of B cell epitopes on small nuclear ribonucleoproteins that react with human autoantibodies as well as with experimentally-induced mouse monoclonal antibodies. *J. Immunol.* **143**, 2560-2566.

Hedley, M. L., Amrein, H. and Maniatis, T. (1995). An amino acid sequence motif sufficient for subnuclear localization of an arginine serine-rich splicing factor. *Proc. Natl. Acad. Sci. USA* **92**, 11524-11528.

- Hetzer, M. and Mattaj, I. W. (2000). An ATP-dependent, Ran-independent mechanism for nuclear import of the U1A and U2B" spliceosome proteins. *J. Cell Biol.* **148**, 293-303.
- Hong, W., Bennett, M., Xiao, Y., Feld Kramer, R., Wang, C. and Reed, R. (1997). Association of U2 snRNP with the spliceosomal complex E. *Nucleic Acids Res.* **25**, 354-361.
- Izaurrealde, E., McGuigan, C. and Mattaj, I. W. (1995). Nuclear localization of a cap-binding protein complex. *Cold Spring Harb. Symp. Quant. Biol.* **60**, 669-675.
- Jády, B. E., Darzacq, X., Tucker, K. E., Matera, A. G., Bertrand, E. and Kiss, T. (2003). Modification of Sm small nuclear RNAs occurs in the nucleoplasmic Cajal body following import from the cytoplasm. *EMBO J.* **22**, 1878-1888.
- Jantsch, M. F. and Gall, J. G. (1992). Assembly and localization of the U1-specific snRNP C protein in the amphibian oocyte. *J. Cell Biol.* **119**, 1037-1046.
- Kambach, C. and Mattaj, I. W. (1992). Intracellular distribution of the U1A protein depends on active transport and nuclear binding to U1 snRNA. *J. Cell Biol.* **118**, 11-21.
- Kambach, C. and Mattaj, I. W. (1994). Nuclear transport of the U2 snRNP-specific U2B" protein is mediated by both direct and indirect signalling mechanisms. *J. Cell Sci.* **107**, 1807-1816.
- Kiss, T. (2001). Small nucleolar RNA-guided post-transcriptional modification of cellular RNAs. *EMBO J.* **20**, 3617-3622.
- Krämer, A. (1996). The structure and function of proteins involved in nuclear pre-mRNA splicing. *Annu. Rev. Biochem.* **65**, 367-409.
- Krämer, A. and Keller, W. (1990). Preparation and fractionation of mammalian extracts active in pre-mRNA splicing. In *Methods Enzymol.*, Vol. 181 (ed. J. E. Dahlberg and J. N. Abelson), pp. 3-19. New York, NY: Academic Press.
- Krämer, A., Legrain, P., Mulhauser, F., Gröning, K., Brosi, R. and Bilbe, G. (1994). Splicing factor SF3a60 is the mammalian homologue of PRP9 of *S. cerevisiae*: the conserved zinc finger-like motif is functionally exchangeable in vivo. *Nucleic Acids Res.* **22**, 5223-5228.
- Krämer, A., Mulhauser, F., Wersig, C., Gröning, K. and Bilbe, G. (1995). Mammalian splicing factor SF3a120 represents a new member of the SURP family of proteins and is homologous to the essential splicing factor PRP21p of *S. cerevisiae*. *RNA* **1**, 260-272.
- Krämer, A., Grüter, P., Gröning, K. and Kastner, B. (1999). Combined biochemical and electron microscopic analyses reveal the architecture of the mammalian U2 snRNP. *J. Cell Biol.* **145**, 1355-1368.
- Kyhse-Anderson, J. (1984). Electrophoretic blotting of multiple gels. *J. Biochem. Biophys. Methods* **10**, 203-209.
- Lamond, A. I. and Carmo-Fonseca, M. (1993). Localisation of splicing snRNPs in mammalian cells. *Mol. Biol. Rep.* **18**, 127-133.
- Lamond, A. I. and Spector, D. L. (2003). Nuclear speckles: a model for nuclear organelles. *Nat. Rev. Mol. Cell Biol.* **4**, 605-612.
- Lerner, E. A., Lerner, M. R., Janeway, C. A., Jr and Steitz, J. A. (1981). Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. *Proc. Natl. Acad. Sci. USA* **78**, 2737-2741.
- Li, H. and Bingham, P. M. (1991). Arginine/serine-rich domains of the su(w^a) and tra RNA processing regulators target proteins to a subnuclear compartment implicated in splicing. *Cell* **67**, 335-342.
- Makarova, O. V., Makarov, E. M., Liu, S., Vornlocher, H. P. and Lührmann, R. (2002). Protein 61K, encoded by a gene (PRPF31) linked to autosomal dominant retinitis pigmentosa, is required for U4/U6.U5 tri-snRNP formation and pre-mRNA splicing. *EMBO J.* **21**, 1148-1157.
- Matera, A. G. (1999). Nuclear bodies: multifaceted subdomains of the interchromatin space. *Trends Cell Biol.* **9**, 302-309.
- Mattaj, I. and Englmeier, L. (1998). Nucleoplasmic transport: the soluble phase. *Annu. Rev. Biochem.* **67**, 265-306.
- Nayler, O., Stratling, W., Bourquin, J. P., Stagljar, I., Lindemann, L., Jasper, H., Hartmann, A. M., Fackelmayer, F. O., Ullrich, A. and Stamm, S. (1998). SAF-B protein couples transcription and pre-mRNA splicing to SAR/MAR elements. *Nucleic Acids Res.* **26**, 3542-3549.
- Nesic, D. and Krämer, A. (2001). Domains in human splicing factors SF3a60 and SF3a66 required for binding to SF3a120, assembly of the 17S U2 snRNP, and prespliceosome formation. *Mol. Cell. Biol.* **21**, 6406-6417.
- Nilsen, T. (1998). RNA-RNA interactions in nuclear pre-mRNA splicing. In *RNA Structure and Function* (ed. R. Simons and M. Grunberg-Manago), pp. 279-307. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Ogg, S. C. and Lamond, A. I. (2002). Cajal bodies and coilin-moving towards function. *J. Cell Biol.* **159**, 17-21.
- Paushkin, S., Gubitz, A. K., Massenot, S. and Dreyfuss, G. (2002). The SMN complex, an assemblyosome of ribonucleoproteins. *Curr. Opin. Cell Biol.* **14**, 305-312.
- Picard, D., Kumar, V., Chambon, P. and Yamamoto, K. R. (1990). Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors. *Cell Regul.* **1**, 291-299.
- Romac, J. M., Graff, D. H. and Keene, J. D. (1994). The U1 small nuclear ribonucleoprotein (snRNP) 70K protein is transported independently of U1 snRNP particles via a nuclear localization signal in the RNA-binding domain. *Mol. Cell. Biol.* **14**, 4662-4670.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schmidt-Zachmann, M. S., Knecht, S. and Krämer, A. (1998). Molecular characterization of a novel, widespread nuclear protein that colocalizes with spliceosome components. *Mol. Biol. Cell* **9**, 143-160.
- Sleeman, J. E. and Lamond, A. I. (1999). Newly assembled snRNPs associate with coiled bodies before speckles, suggesting a nuclear snRNP maturation pathway. *Curr. Biol.* **9**, 1065-1074.
- Sleeman, J. E., Ajuh, P. and Lamond, A. I. (2001). snRNP protein expression enhances the formation of Cajal bodies containing p80-coilin and SMN. *J. Cell Sci.* **114**, 4407-4419.
- Spector, D. L. (1993). Macromolecular domains within the cell nucleus. *Annu. Rev. Cell Biol.* **9**, 265-315.
- Spector, D. L., Fu, X.-D. and Maniatis, T. (1991). Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO J.* **10**, 3467-3481.
- Stanek, D., Rader, S. D., Klingauf, M. and Neugebauer, K. M. (2003). Targeting of U4/U6 small nuclear RNP assembly factor SART3/p110 to Cajal bodies. *J. Cell Biol.* **160**, 505-516.
- Will, C. L. and Lührmann, R. (1997). SnRNP structure and function. In *Eukaryotic mRNA processing* (ed. A. R. Krainer), pp. 130-173. Oxford, UK: IRL Press.
- Will, C. L. and Lührmann, R. (2001). Spliceosomal UsnRNP biogenesis, structure and function. *Curr. Opin. Cell Biol.* **13**, 290-301.
- Will, C. L., Schneider, C., MacMillan, A. M., Katopodis, N. F., Neubauer, G., Wilm, M., Lührmann, R. and Query, C. C. (2001). A novel U2 and U11/U12 snRNP protein that associates with the pre-mRNA branch site. *EMBO J.* **20**, 4536-4546.
- Will, C. L., Urlaub, H., Achsel, T., Gentzel, M., Wilm, M. and Lührmann, R. (2002). Characterization of novel SF3b and 17S U2 snRNP proteins, including a human Prp5p homologue and an SF3b DEAD-box protein. *EMBO J.* **21**, 4978-4988.
- Yu, Y. T., Shu, M. D. and Steitz, J. A. (1998). Modifications of U2 snRNA are required for snRNP assembly and pre-mRNA splicing. *EMBO J.* **17**, 5783-5795.
- Zhao, X. and Yu, Y. T. (2004). Pseudouridines in and near the branch site recognition region of U2 snRNA are required for snRNP biogenesis and pre-mRNA splicing in *Xenopus* oocytes. *RNA* **10**, 681-690.